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HEXOKINASES OF SPINACH LEAVES*

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Abstract—Two soluble hexokinases and a particulate hexokinase have been separated and partially purified from spinach leaves. One of the soluble hexokinases showed a high affinity for glucose (K_m = 63 μM) which was far greater than that for fructose (K_m = 9.1 mM). However, with saturating fructose the activity was twice that with saturating glucose. The particulate hexokinase showed kinetic properties similar to those of this soluble hexokinase. The second soluble hexokinase was distinct in that it was much more active with fructose than with glucose at all concentrations tested, although the K_m values for these hexoses (210 μM and 71 μM respectively) were similar. The activity of this hexokinase was stimulated by the monovalent cations K^+ and NH_4^+.

INTRODUCTION

Plant hexokinases (ATP: D-hexose 6-phosphotransferase, EC 2.7.1.1) have been much less investigated than their yeast and mammalian counterparts. Four isoenzymes have been identified in mammalian tissues, and at least two of these are to a considerable extent particulate, apparently associated with the outer mitochondrial membrane [1]. The isoenzymes display clear differences with respect to their relative affinities for glucose and fructose [1]. Initial investigations on plant hexokinases showed that these too were distributed between soluble and particulate fractions [2], and that different enzyme forms with markedly contrasting affinities for glucose and fructose existed [3]. Subsequent investigations with a variety of non-photosynthetic plant tissues have established the presence of non-specific hexokinases, occurring in both soluble and particulate fractions, and specific glucokinase and fructokinase enzymes in soluble fractions [4–9].

The hexokinases of the photosynthetic tissues of plants have been essentially neglected. Since Saltman [2] originally reported that spinach leaves contained both soluble and particulate hexokinase activities, the only leaf enzymes subsequently investigated were from parasitic plants [10]. However, research on the carbohydrate metabolism of leaves in darkness has raised questions concerning the nature and distribution of the hexokinase activities in leaves [11–13]. An investigation of spinach-leaf hexokinase was therefore initiated.

RESULTS AND DISCUSSION

Soluble hexokinases

When a sample of the soluble protein from spinach leaves was chromatographed on DEAE-cellulose, four distinct peaks of enzyme activity were obtained (Fig. 1). For each of the first three peaks, the activity was of the same order of magnitude whether 5 mM glucose or 5 mM fructose was provided as substrate, but the last-eluted peak was far more active with fructose than with glucose. Peaks I–III therefore appeared to be non-specific hexokinases, while peak IV was concluded to be a fructokinase. A pattern of four peaks was also obtained by Turner *et al.* [8] following DEAE-cellulose chromatography of a preparation from pea seeds, but in those experiments the first two peaks were somewhat less active with fructose than with glucose, while the last two peaks showed a marked preference for fructose.

Two of the spinach-leaf hexokinases (peaks II and IV) were further purified and some kinetic properties compared. The soluble hexokinase II displayed an affinity for glucose (K_m = 63 μM) over 100 times that for fructose (K_m = 9.1 mM) and in this respect could be looked upon as a glucokinase comparable to that identified in pea seeds [8]. However, unlike the pea-seed enzyme, the activity of this spinach-leaf hexokinase with fructose was twice that with glucose when near-saturating concentrations of the respective hexoses were compared.

The response of the spinach-leaf soluble hexokinase IV to increasing concentrations of glucose or fructose contrasted markedly with that of hexokinase II. Hexokinase IV clearly preferred fructose at all hexose concentrations, despite the fact that the affinity for fructose (K_m = 210 μM) was only one-third that for glucose (K_m = 71 μM). The maximum activity with fructose was over 15 times that observed with glucose. Other differences between the hexokinases II and IV were also observed: hexokinase IV had a greater affinity for Mg^{2+} (Fig. 2), and according to position of elution from a column of Sephadex G-200 (not shown), it had a significantly greater molecular size than hexokinase II. However, both had similar broad pH optima in the region of 7.5–8.5.

Spinach-leaf hexokinase IV was similar to the comparable pea-seed enzyme [9] with respect to (a)

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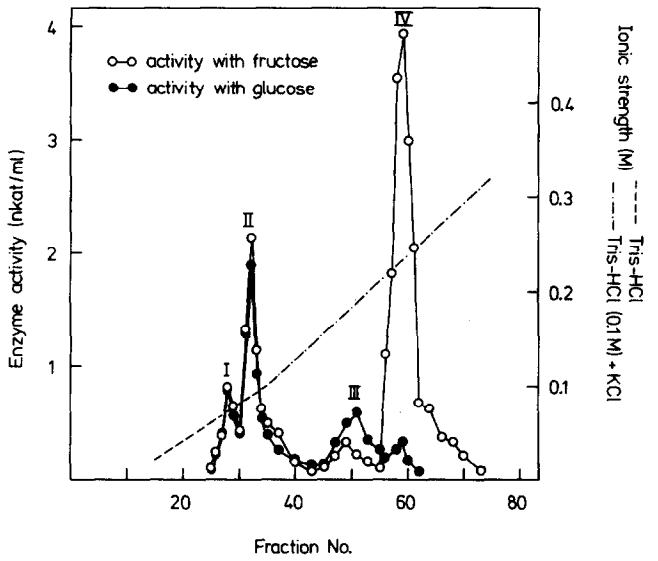

Fig. 1. DEAE-cellulose chromatography of the hexokinase activity in a 30–80% ammonium sulphate fraction from spinach leaves. For details of the procedure and assay of the enzyme see Experimental.

inhibition by high concentrations of fructose or Mg^{2+} ([9], and Fig. 2; the activity of the spinach-leaf enzyme with 17.5 mM fructose was half that with 1 mM fructose), (b) dependence on a monovalent cation for full activity (Table 1, [9]), and (c) a far greater maximum activity with fructose than with glucose. However, in contrast to the pea-seed enzyme, spinach-leaf fructokinase IV had a greater affinity for glucose than for fructose (see above).

Particulate hexokinase

Experiments with whole-leaf extracts confirmed the observation of Saltman [2] that about one-third of the total hexokinase in spinach leaves is particulate. When an unfractionated leaf extract containing 81.6 nkat hexokinase activity was centrifuged, only 46.2 nkat of activity was recovered in the supernatant. Three washings of the precipitate with buffer (see Experimental) released a further 6.7, 5.4, and 3.1 nkat activity, respectively. Finally, a fourth wash with buffer containing 1% Triton X-100 released a further 14.3 nkat, representing 19% of the recovered activity.

The response of this particulate enzyme to the concentration of glucose or fructose was almost identical to that of the soluble hexokinase II, with the exception that the maximum activity with fructose was not so notably higher than that with glucose. There was also little to distinguish between the effects of Mg^{2+} concentration and pH on the particulate hexokinase and on the soluble hexokinase II (not shown). This similarity of a particulate hexokinase to a soluble form of the

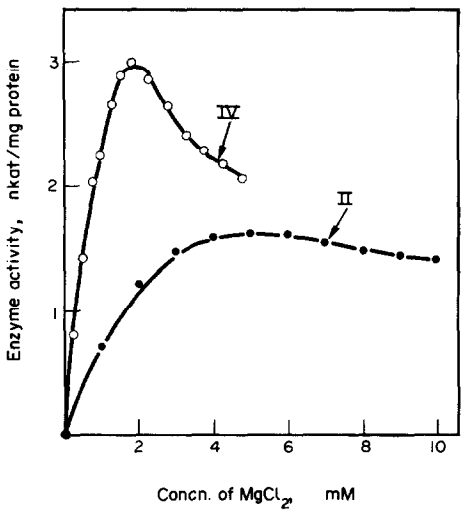

Fig. 2. Effect of Mg^{2+} concentration on the activities of the spinach-leaf soluble hexokinases II and IV. The hexokinases were partially purified as indicated under Experimental. Reaction mixtures were as described in the Experimental, with glucose (II) or fructose (IV) as substrate, except the concentration of $MgCl_2$ was varied as shown, and 30 mM KCl was included for IV.

Table 1. Effect of cations on the activity of the soluble hexokinase IV after filtration through Sephadex G-200

Salt	Enzyme activity
	nkat/mg protein
None	0.62
10 mM KCl	1.02
30 mM KCl	1.97
10 mM NaCl	0.68
30 mM NaCl	0.70
10 mM $(NH_4)_2SO_4$	0.80
30 mM $(NH_4)_2SO_4$	1.30

The gel filtration procedure and the composition of reaction mixtures are described in the Experimental; activity was measured with 0.1 ml enzyme preparation and with fructose as substrate.

enzyme was not altogether unexpected, since it has already been documented for the mammalian hexokinases [1].

The location of the particulate hexokinase is unclear. Preliminary experiments indicated that both mitochondria and chloroplasts contained bound hexokinase, but the activity found associated with the chloroplasts was too low to exclude the possibility that it was due to mitochondrial contamination. Baijal and Sanwal [10] also concluded that leaf mitochondria, but not chloroplasts, possess hexokinase activity.

The amounts of free glucose and fructose found in leaves ([14], G. Vester, personal communication) are consistent with *in vivo* concentrations of *ca* 6 mM and 2 mM, respectively. It is therefore not difficult to envisage that a principal physiological role of the leaf hexokinases reported here is the phosphorylation of these hexoses prior to their further metabolic utilization.

EXPERIMENTAL

Materials. Spinach (*Spinancia oleracea* L., var. Früremona) was grown in a growth chamber at 15° with a 10-hr day (200 W/m²)/14-hr night. Glucose and fructose were obtained from Sigma (München). Other biochemicals and the auxiliary enzymes were purchased from Boehringer (Mannheim). DEAE-Cellulose type 23SS was supplied by Serva (Heidelberg), and Sephadex products were obtained from Pharmacia GmbH (Frankfurt).

Partial purification of soluble hexokinases. Spinach leaves (100 g) were homogenized with 250 ml buffer A (50 mM Tris–HCl, pH 8.0, containing 5 mM $MgCl_2$, 1 mM EDTA and 1 mM β-mercaptoethanol), and the homogenate filtered through Miracloth. The filtrate was fractionated by addition of solid $(NH_4)_2SO_4$: ppt. formed at 30% salt satn was centrifuged (30 000 g, 15 min) and discarded. The salt satn of the supernatant was raised to 80% and the ppt. which formed was collected by centrifugation, dissolved in 25 ml buffer B (as buffer A, but with 20 mM Tris–HCl), and dialysed against 2 l this buffer overnight. The prepn was clarified by centrifugation, then applied to a column of DEAE-cellulose (25 cm × 2.0 cm²) previously equilibrated with buffer B. After washing with one bed vol. of buffer B, enzyme activity was eluted with 60 ml of a linear gradient of Tris–HCl between 20 and 100 mM, followed by 240 ml of a linear gradient of KCl between 0 and 400 mM in 100 mM Tris–HCl. $MgCl_2$, EDTA and β-mercaptoethanol were present throughout at the concns present in buffer A. Fractions of 3 ml were collected. Four peaks of hexokinase activity were obtained, and numbered I–IV according to order of elution (Fig. 1). Fractions containing the bulk of the activity in each peak were combined, and peaks II and IV were then separately further purified by the following procedure: each was pptd by addition of $(NH_4)_2SO_4$ to 80% satn, redissolved in 4 ml buffer B, then filtered through a column of Sephadex G-200 (90 cm × 1.8 cm²) using buffer B for equilibration and elution. Fractions of 3 ml were collected, and those containing activity were combined and the protein pptd and redissolved as described above. The prepn was dialysed overnight, then applied to a column of DEAE-Sephadex A-50 (10 cm × 1.8 cm²), previously equilibrated with buffer B. A gradient of 160 ml KCl (0–400 mM) was used to elute activity, and fractions containing the bulk of the activity were combined and stored. All operations were at 2–4°. Hexokinase II lost little activity during storage for 2 weeks at 4°, but hexokinase IV lost over 50%. However, this was reduced to 20% by storage at −20° in 20% glycerol.

Partial purification of particulate hexokinase. A Miracloth filtrate of an extract from 100 g leaves obtained as described above, was centrifuged at 4000 *g* for 45 min. The supernatant was reserved. The ppt. was washed ×3 with 20 ml buffer B; each washing was followed by centrifugation (20 000 *g*, 15 min.) and each supernatant reserved. Finally, the ppt. was stirred for 30 min with 20 ml buffer B containing 1% Triton X-100, then centrifuged at 40 000 *g* for 30 min. The clear green supernatant obtained was reserved, and all 5 supernatants assayed for hexokinase activity.

The supernatant from the Triton extraction was further purified by chromatography on a DEAE-cellulose column (20 cm × 2.0 cm²) as described above, except all buffers contained 1% Triton X-100. During this step, activity was separated from chlorophyll-containing material. Active fractions were combined, concd to 5 ml by ultrafiltration over a Diaflo XM50 membrane, then dialysed overnight against 1 l. buffer B. The prepn was finally chromatographed on DEAE-Sephadex A-50 as described above. Active fractions were combined, and protein was pptd (by adding $(NH_4)_2SO_4$ to 80% satn), then redissolved in 3 ml buffer B. All operations were at 2–4°.

Assay of hexokinase activity. Enzyme activity was determined by following $NADP^+$ reduction in the presence of excess glucose-6-phosphate dehydrogenase (G-6-P dehydrogenase). Reaction mixtures contained, in a final vol. of 1 ml, 100 μmol Tris–HCl buffer (pH 8.0), 5 μmol $MgCl_2$, 0.7 unit G-6-P dehydrogenase, spinach-leaf hexokinase prepn, and either 5 μmol glucose or 5 μmol fructose plus 1.5 units phosphoglucose isomerase. Reactions were started by adding 2.5 μmol ATP, and the change in absorbance at 366 nm measured with a photometer at 30°. Protein was determined according to Lowry *et al.* [15] or by the biuret procedure.

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